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14. ABSTRACT Prostate cancer is the most commonly diagnosed and second most deadly cancer in North American men and the blockade of androgen action through the AR has been the cornerstone of systemic therapy of prostate cancer. The failure of AR receptor antagonists results in higher levels of AR protein which promotes the development of androgen-independent prostate cancer. Originally we proposed the utilization of micro (mi) RNAs to blockade the expression of AR in prostate carcinoma cells. We have identified a miRNAs that can repress the AR protein synthesis in prostate carcinoma cells. Our long-term goals are to identify naturally occurring miRNAs that have potential to block the activity of AR and to improvise their efficacy by rational designing to provide novel 'AR Antagonist miRNAs'.				
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INTRODUCTION:

Prostate cancer is the most commonly diagnosed and second most deadly cancer in North American men. Androgen receptor (AR) protein plays an important role in the development and progression of prostate cancer. Blockade of androgen action through the AR has been the cornerstone of systemic therapy of prostate cancer. However, the effectiveness of this therapy is rather transient which inevitably fails and tumor growth resumes despite androgen blockade. The failure of AR receptor antagonists results in higher levels of AR which is one of the causative factors of the development of androgen-independent prostate cancer. We proposed the utilization of micro (mi) RNAs to blockade the expression of AR in prostate carcinoma cells. Our goals are to identify naturally occurring miRNAs that affect the activity of AR and to improve their efficacy by rational RNA-RNA interactions (between miRNA and 3' mRNA region of AR) to provide novel 'therapeutic miRNAs' which can target the expression of AR.

BODY:

Task 1. To examine if androgen receptor (AR) translation is modulated by a naturally occurring hsa-mir-183 microRNA (miRNA) and to validate that the 3'UTR of AR is a bona fide target of miRNA using chimeric luciferase reporter constructs (Months 1-12).

Task 1a Proposed Work: Design and commercial acquisition and subsequent overexpression of synthetic miRNAs by transient transfections into LNCaP cells and investigation of AR expression levels by Western blot using anti-AR antibodies. Confirmation that hsa-miR-183 is involved in AR translational control will be achieved (Months 1-3).

We have cloned genomic regions coding miRNA and putative cis elements into lentiviral vectors as well as in eukaryotic expression vector pcDNA to examine their effect in AR protein repression in prostate carcinoma cells LNCaP (see below).

Task 1b Proposed Work: Development of multiple test and control luciferase reporter expression constructs, each with an engineered 3' untranslated region (miRNA targeted sequences) of AR cloned downstream of the stop codon of luciferase cDNA. Chimeric luciferase and appropriate control reporter constructs will be ready (months 1-6).

We have synthesized the luciferase reporter with androgen receptor 3' UTR cloned in the downstream of the stop codon. We have chimeric luciferase and its function was tested in transiently transfected cell culture system. Results are shown below;

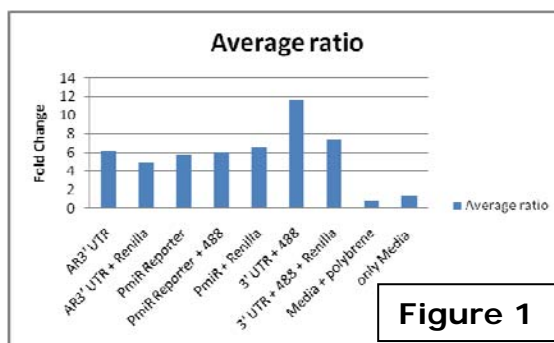


Figure 1

Figure 1 Testing of firefly luciferase containing 3' UTR of androgen receptor in transiently transfection: Firefly luciferase with or without 3' UTR of AR along with appropriate controls were transiently transfected in CHO cells using polybrene. Cells were DMSO shocked 8 hours posttransfection and cells were harvested at 48 hours. Total protein lysates were prepared and equal amount of proteins from the each transfected wells were used to

perform dual luciferase assay. To repress the expression of firefly luciferase expressing AR 3' UTR we cotransfected pcDNA miR-488. All assays were performed in triplicates and ratio of firefly and renilla luciferase were plotted. pcDNA clone expressing miR-488 was unable to repress the expression of firefly luciferase as shown in "3'UTR+488" in Figure 1. Nonetheless, this experiment established that our chimeric

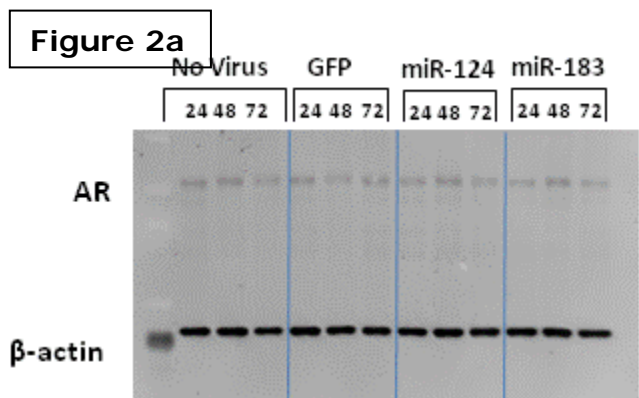
firefly luciferase/AR 3'UTR is able to express luciferase in heterologous CHO cell culture system. Therefore, we successfully achieved the Task 1b.

Task 1c Proposed Work: Confirmation that AR 3' UTR sequences containing chimeric luciferase reporter constructs are responsive to ectopically expressed synthetic miRNAs. Optimization of reporter assays, evaluation of target specificities and interactions between target sequences and miRNAs will be achieved (Months 6-8).

Currently, we are in the process of optimization of chimeric firefly luciferase and renilla luciferase in order to test the effect of ectopically expressing miRNAs. Also, we are optimizing transfection of pcDNA miR-124a, pcDNA-183 and pcDNA-488 with chimeric firefly luciferase in transient transfection system. In addition, we will generate stably expressing luciferase under Puromycin selection. This would enable us to expedite our transfection experiments.

Additional Work Done: We have generated Hsa-miR-183, Hsa-miR-124a and Hsa-miR-488 expressing lentiviruses for stable expression in LNCaP cells. During this period we tested if stable overexpression of miRNAs in LNCaP cells can target the AR mRNA to modulate the protein synthesis.

We have cloned the coding regions encompassing genomic sequences into Lentiviral vectors. Virus expressing miR-183, miR-124a and miR-488 were generated in 293 T cells. Viruses were purified and concentrated utilizing established protocols. The purified viruses were dissolved in RPMI 1640 medium and LNCaP cells were transduced using these viruses. RNA and protein lysates were produced from these cells to perform qRT-PCR and Western Blot to detect the effect of miRNAs on the RNA and protein levels of Androgen receptor respectively. No significant knockdown of AR protein was observed in LNCaP cells as compared to no virus



lanes. It is possible either these miRNA are not being produced by lentiviruses or AR is not a target of hsa-miR-124 and hsa-miR-183 miRNAs when transfected individually. To check if viral system is producing hsa-miR-124 and hsa-miR-183 expression we performed Northern Blot, however unable to detect the expression of these miRNAs. Ribonuclease protection assays will be done to establish if miRNAs are being expressed from lentiviruses.

Figure 2 A: Western Blot of AR protein prepared from cells expressing miR-124 and miR-83: A time course of 24 hours, 48 hours and 72 hours was done to study the affect of ectopic miR expression on endogenous LNCaP AR. NV- No Virus transduced in LNCaP cells, GFP – cells were transduced with green fluorescent protein expressing lentivirus, miR-124 and miR-183 corresponds to miRNAs. Beta actin was used as loading control.

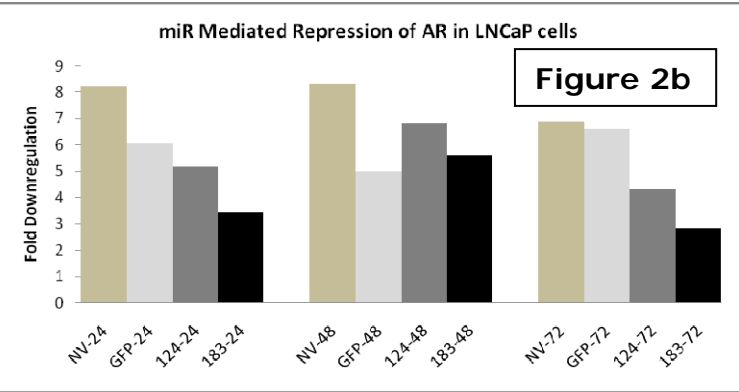


Figure 2B: Quantitation of AR protein in lentiviral mediated LNCaP cells: The histogram was plotted on the values of AR protein bands shown in Figure 2a as determined by densitometric scans and image quant program. The miR-124 and miR-183 shows a reduction in AR protein level at 72 hours as compared to 24 hours. Currently, we are in the process of repeating the experiment.

Figure 3a and 3b: These Figures are essentially the similar to experiment shown in Figures 2a and 2b except in this experiment we tested lentiviral miR-488 expression. As shown in the panel 3b ‘488-72’ marked reduction of AR protein was observed. Currently, we are in the process of repeating the experiments.

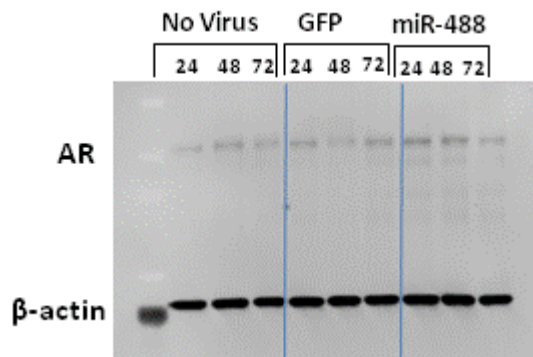


Figure 3a

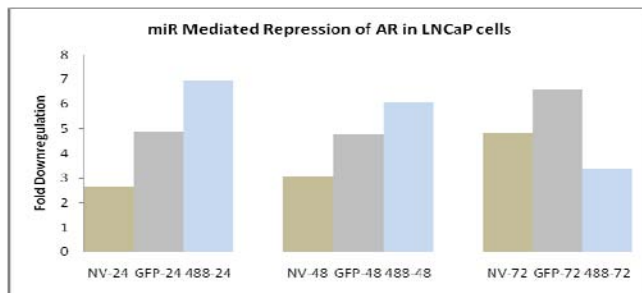


Figure 3b

Task 1d Proposed Work: Rational design and testing of designer miRNAs that can modulate the expression of AR 3' UTR containing chimeric luciferase reporter constructs. (Months 8-10).

Work Done: All the experiments stated above will be repeated and a miRNA which can maximally repressed the expression of endogenous LNCaP AR and also represses the firefly luciferase expression in CHO will be rationally ‘redesigned’. This will be done by increasing or decreasing the RNA:RNA base-pairing interaction between the 3' AR UTR target and miRNA. So far, miR-488 appears to be a better candidate miRNA that has potential to knockdown the expression of miRNA at greater levels.

Task 2.

Proposed Work: Development of an inducible miRNA based cell culture model system to target translational knockdown of endogenous androgen receptor in prostate carcinoma LNCaP cells.

Work Done: Since last report, we got one step closer in the identification of a miRNA which can target the expression of AR. However, we are not able to confirm miRNA mediated effect of AR in cell based assays. Once we identified a miRNA using above mentioned techniques, and identify its role in cellular assays, we will subclone that miRNA in inducible expressing systems in and test for it functionality in the prostate carcinoma cell culture system. This specific aim is depend on the success of Task 1d.

PROBLEMS: The funds from this grant mechanism are ended and it is in “no cost” extension mode. We have applied for more funds from PCRP funding mechanism to continue to work on this project. However, currently we have very limited resource to continue to fund this project. A final report stating all the accomplishment will be submitted in 2009. However, below we describe interim accomplishment of the project.

KEY RESEARCH ACCOMPLISHMENTS: Lentiviral expression vectors expressing miR-124, miR-183 and miR-488 are available. We also have chimeric firefly luciferase with AR 3' UTR construct. As a proof of principle we have tested the expression of chimeric luciferase in CHO cells. The optimization of miR and chimeric firefly luciferase will be pursued, if we are able to generate sufficient funds.

REPORTABLE OUTCOMES: As of now, we do not have reportable outcomes from this funding. However, to continue to build upon the knowledge and resources we have gained during this period, I have applied for New Investigator Award from PCRP.

CONCLUSION: We have developed reporter constructs expressing multiple miRNAs and have opportunity to test for their efficacy in AR inhibition in prostate cancer cells. The experimental identification of miRNAs which maximally repress the translational activity of the AR and further improvisation in their design will allow us to develop a subset of “AR antagonist miRNA”.

SO WHAT: The knowledge gained from our efforts so far indicates that our project is heading in right direction. However, we are unable to sustain the momentum due to lack of funds and personnel. We would be able to use our constructs, if resources allow, in blockading the expression of AR in prostate carcinoma cell model systems, and perhaps in mouse model of prostate cancer.